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THE REACTIVITIES OF TYROSINE AND TRYPTOPHAN RESIDUES IN LIPID-BOUND CYTOCHROME b_5 *

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Summary

Purified cytochrome b_5 from rabbit liver microsomes was bound to liposomes prepared from microsomal lipids. Tyrosyl and tryptophyl side chains of the protein were modified by water-soluble reagents and the reactivities of these amino acid residues in the liposome-bound cytochrome b_5 were compared to those of the free protein. At pH 13, 80% of the tyrosines in lipid-free cytochrome b_5 ionized immediately, whereas in the lipid-bound protein only 65% ionized within the first minute. In contrast, acetylation with acetylimidazole resulted in the conversion of all 5 tyrosine groups of lipid-free as well as lipid-bound cytochrome b_5 into *O*-acetylated derivatives, which upon treatment with hydroxylamine were completely deacetylated. Reaction with *N*-bromosuccinimide revealed that only 60% of the 4 tryptophan residues present in cytochrome b_5 were accessible to the reagent in the lipid-bound protein, although all tryptophans could be modified in lipid-free cytochrome b_5.

It was concluded that the two tyrosines in the region linking the protein to the membrane are not shielded by the lipid bilayer, but that of the three tryptophans in the same region one is completely buried in the membrane, whereas the remaining two tryptophans may be both partly exposed to the solvent or alternatively, one may be partially and the other completely exposed.

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Abbreviations: cytochrome *t*-b_5, cytochrome b_5 purified after trypsin digestion of microsomes; cytochrome *d*-b_5, cytochrome b_5 purified after detergent solubilization of microsomes; lipo-cytochrome b_5, cytochrome b_5 bound to liposomes.

Introduction

Cytochrome b_5 is an amphipathic protein of the endoplasmic reticulum. In rabbits it consists of a hydrophilic domain of about 90 amino acids which contains the heme and protrudes into the cytoplasm, and of a predominantly hydrophobic domain of about 40 amino acids which links the protein to the membrane matrix [1]. The entire polypeptide chain contains 141 amino acid residues [1]. Tryptic digestion of the microsomes yields only the hydrophilic part, still catalytically active but unable to bind to membranes. Treatment of the microsomes with detergents solubilizes the complete molecule which can be purified and bound to microsomes [2,3] or liposomes prepared from phosphatidylcholine [4—6] or microsomal lipids. These lipoproteins constitute a simplified membrane system which should facilitate studies on protein-lipid interactions.

It is clear that the hydrophobic region of cytochrome b_5 is implicated in the binding to the membrane, but it is not yet known to what extent it is actually immersed in the lipid bilayer. The experiments described below were concerned with this question. They compare the chemical reactivities of the tyrosines and the tryptophans in the liposome-bound cytochrome b_5 to those of the free cytochrome b_5.

Our hypothesis was that in lipo-cytochrome b_5 the nonpolar segment is inserted into the phospholipid bilayer which shields it from attack by water-soluble reagents. Therefore, the reactivities of amino acids in the non-polar region of the lipid-bound polypeptide should be less than those in the free protein.

Materials and Methods

Sephadex G 25, G 75 and G 200 were products of Pharmacia. Ultrogel AcA 34 was manufactured by LKB. *N*-bromosuccinimide was purchased from Merck and recrystallized three times from water. *N*-acetylimidazole was obtained from Serva.

Microsomes were prepared as described previously [7]. Lipids were extracted from rat liver microsomes according to Holtzman and Gillette [8] and stored in a chloroform solution at −30° C for up to four weeks.

Liposomes were prepared by the method of Huang [9]: 5—20 mg/ml of the lipids were swollen in 0.02 M Tris acetate/0.0002 M EDTA/0.1 M NaCl (pH 8.1) and sonicated under nitrogen for 30 min. The formation of bilayers was checked by electron microscopy.

Liposomes containing potassium ferricyanide were prepared by sonicating the lipids in 1 M $K_3[Fe(CN)_6]$ dissolved in 0.05 M Tris acetate/0.01 M EDTA (pH 8.1) and separated from external ferricyanide by passage through Sephadex G 25 equilibrated with the same buffer.

Cytochrome *t*-b_5 was purified by the trypsin digestion method of Schnellbacher and Lumper [10]. Cytochrome *d*-b_5 was isolated by the detergent solubilization procedures of Spatz and Strittmatter [1] and Ozols [11] with the following modifications. Washing of the microsomes with 1 M NaCl was omitted. Three gel filtrations through Sephadex G 75 in 0.25 M KSCN/0.25%

deoxycholate were performed. Judging from disc gel electrophoresis, the protein was more than 95% pure. Most preparations had a ratio of A_{413nm}/A_{280nm} = 2.65. Lipo-cytochrome b_5 was prepared by incubating cytochrome d-b_5 in 0.02 M Tris acetate/0.0002 M EDTA/0.02 M $MgCl_2$ or 0.1 M NaCl (pH 8.1) with freshly sonicated liposomes. The mixture was incubated at 37°C for 30 min, then left standing overnight at 4°C. The lipoprotein was isolated by filtration through Sephadex G 200, equilibrated with 0.02 M Tris acetate/0.0002 M EDTA (pH 8.1) or through Ultrogel AcA 34 in 0.05 M Tris acetate/0.01 M EDTA (pH 8.1). Elution was performed using the equilibration buffer.

The hydrophobic peptide bound to liposomes was prepared from lipo-cytochrome b_5 by trypsin digestion according to Spatz and Strittmatter [1]. The lipid-bound peptide was isolated by passage through the same Sephadex G 200 column as was used for the preparation of lipo-cytochrome b_5. Assuming that the molar ratio of cytochrome b_5 to phospholipids was the same in the original lipo-cytochrome b_5 and the resulting lipid-bound hydrophobic peptide, the concentration of the latter could be calculated by determination of phosphorus.

Alkaline hydrolysis of oxidized lipo-cytochrome b_5 with 2 M $Ba(OH)_2$ was carried out after exhaustive dialysis against water [12]. A crude NADH-cytochrome b_5-reductase was obtained by lysosomal digestion of rat liver microsomes as described by Takesue and Omura [13].

Analytical Procedures

Cytochrome b_5 was assayed in a Pye-Unicam SP 8000 spectrophotometer assuming an extinction coefficient for the oxidized Soret band of 117000 [1] at 413 nm.

The enzymatic reduction of cytochrome d-b_5 and lipo-cytochrome b_5 was assayed according to [13]. Lipid phosphorus was determined by the procedure of Bartlett [14]. The molecular weight of the phospholipids was assumed to be 800. Since about 85% of the microsomal lipids are phospholipids [15], the results from the phosphorus determinations were taken as representing all the microsomal lipids. Deoxycholate was assayed according to [16].

The concentration of ferricyanide enclosed by liposomes carrying cytochrome d-b_5 was determined spectrophotometrically after enzymatic reduction with NADH and NADH-cytochrome b_5-reductase as described in ref. 13 or after chemical reduction with sodium dithionite. The reduction of trapped ferricyanide was initiated by addition of deoxycholate (final concentration 0.1%) and followed by the subsequent decrease of the absorption at 420 nm. An extinction coefficient of 1.02 $cm^{-1} \cdot mM^{-1}$ was used [13].

Tyrosine was titrated as described by Inada [17]. The ionic strength was maintained at 0.52 while the pH varied between 7 and 13. Difference spectra of alkaline minus neutral solutions between 270 nm and 340 nm were recorded with an Aminco-Chance DW 2 spectrophotometer immediately after the addition of alkali and at 45-min intervals for 180 min. The degree of ionization at a given pH was calculated using the formula:

$$\frac{(A_{295\,nm} - A_{315\,nm})t = 1\text{ min, pH } x}{(A_{295\,nm} - A_{315\,nm})t = 180\text{ min, pH } 13}$$

($A_{295nm} - A_{315nm}$) at t = 180 min, pH 13 corresponded to the ionization of 5 mol tyrosine per mol cytochrome b_5. This was calculated using an extinction coefficient of 2300 $cm^{-1} \cdot M^{-1}$ for the phenoxide ion [17].

In several instances, the shape of the difference spectra changed during these 3 h. In these cases ($A_{295nm} - A_{315nm}$) t = 120 min (pH 13) was used instead. Generally, the final value of $A_{295nm} - A_{315nm}$ was reached between 45 and 90 min after the addition of alkali.

The tyrosines were acetylated with *N*-acetylimidazole according to Riordan [18]. The resulting *O*-acetylated protein was separated from the reagent by filtration through Sephadex G 25. The number of tyrosine residues acetylated per mol protein was determined by incubating the acetylated protein with 0.5 M NH_2OH at pH 7.5 for 1 h and determining the increase of absorbance at 280 nm. For the calculation of the extent of deacetylation a molar extinction coefficient of 1160 $cm^{-1} \cdot M^{-1}$ was used [18].

Tryptophan oxidation with *N*-bromosuccinimide was carried out by the method of Witkop and Spande [19]. Cytochrome *d*-b_5 and lipo-cytochrome b_5 were denatured with 8 M urea, pH 4 or 6.6. 4.8 or 9.6 mM *N*-bromosuccinimide was added in 10 µl amounts to 3 ml samples of 4—12 µM protein solutions. 5—10 min after each addition the change in absorbance at 280 nm was measured by difference or by absolute spectrophotometry in an Aminco Chance DW 2 spectrophotometer until the absorbance at 280 nm remained constant.

Results

(a) The binding of cytochrome b_5 to liposomes

As already shown in several laboratories [4,5], the incubation of cytochrome *d*-b_5 with liposomes at physiological pH results in the formation of liposome-bound cytochrome *d*-b_5, provided that the liposomes are not prepared from negatively charged lipids [6]. In our experiments generally 60—80% of the protein incubated bound to liposomes prepared from microsomal lipids and could be separated from unbound protein by gel filtration through Sephadex G 200 or Ultrogel AcA 34. As shown in Fig. 1, these gels exclude liposomes and lipo-cytochrome b_5 but retain cytochrome *d*-b_5 which in aqueous buffers has an apparent mol. wt. of 120000 [1]. Depending on the relative amounts of protein and lipid incubated, the ratio of protein to phospholipid in the isolated lipoprotein varied from 1 : 2 to 1 : 5 (w/w).

Lipo-cytochrome b_5 in concentrations up to 30 nmol/ml was only slightly opalescent even after freezing and thawing. Therefore, spectrophotometry in the ultraviolet region was possible.

In order to determine whether the protein retains the original conformation of the hydrophilic domain after binding to liposomes, the rate of reduction of lipo-cytochrome b_5 by a crude soluble NADH-cytochrome b_5-reductase preparation was compared to that of cytochrome *d*-b_5. At 2, 4 and 6 µM concentrations lipo-cytochrome b_5 was reduced by NADH via NADH-cytochrome b_5-reductase at essentially the same rate as cytochrome *d*-b_5. That is: lipo-cytochrome b_5 interacted with the reductase as efficiently as did cytochrome *d*-b_5 (Fig. 2).

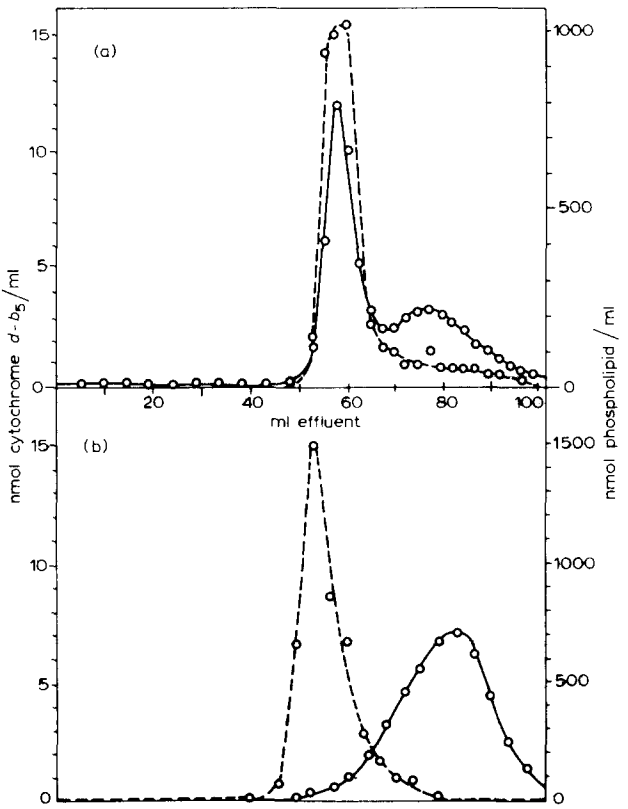

Fig. 1. Elution profiles of lipo-cytochrome b_5, cytochrome d-b_5 and liposomes. (a) 240 nmol cytochrome d-b_5 in 4 ml 0.05 M Tris acetate/0.01 M EDTA/0.1 M NaCl (pH 8.1) were incubated with 13 mg sonicated lipid for 30 min at 37°C and subsequently passed through Ultrogel AcA 34 (column dimensions 30 × 3 cm diameter) in 0.05 M Tris acetate/0.01 M EDTA (pH 8.1). ○———○, cytochrome b_5; ○- - - - -○, phospholipid. (b) 4 ml 40 μM cytochrome d-b_5 and 4 ml 6 mM sonicated lipid were passed separately through the same column as was used in (a). ○———○, cytochrome d-b_5; ○- - - - -○, phospholipid.

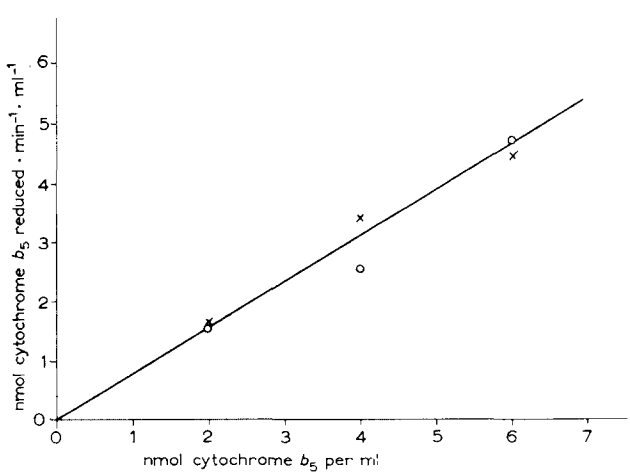

Fig. 2. Reduction rate of lipo-cytochrome b_5 and cytochrome d-b_5 with NADH-cytochrome b_5-reductase. Cytochrome d-b_5 and lipo-cytochrome b_5 at three different concentrations were reduced by 20 μg crude NADH-cytochrome b_5-reductase in 1 ml 10^{-4} M NADH/0.1 M phosphate buffer (pH 7.5). Reduction of cytochrome b_5 was followed spectrophotometrically at 423 nm. —X—X—, cytochrome d-b_5; —○—○—, lipo-cytochrome b_5.

(b) Modification of the tyrosines

Cytochrome b_5 contains 5 tyrosyl residues. Three of these are situated in the hydrophilic part corresponding to cytochrome t-b_5 in positions 10, 11, and 34 [20]. Studies by Huntley [21] on the reactivities of these tyrosines revealed that up to pH 12 tyrosine 34 does not ionize readily but can be acetylated and iodinated. Tyrosines 10 and 11 are easily accessible to all kinds of reagents as could be expected from the X-ray structure determined by Mathews [22]. The two other residues belong to the hydrophobic peptide, the primary structure of which is not yet known. The experiments described below compare the exposure to OH^- of these tyrosines of cytochrome d-b_5 to that of liposome-bound cytochrome d-b_5.

Since the behaviour of liposomes under extremely alkaline conditions is not known, a control experiment was carried out to test whether bilayer structure is retained at pH 13 at least for short periods of time. This could be shown by using liposomes containing a trapped solute and determining the amount of solute retained by liposomes after exposure to alkali. Liposomes containing potassium ferricyanide were incubated with cytochrome d-b_5 at 37°C, subsequently brought to pH 13 and 1 min after the addition of alkali passed through Sephadex G 25 equilibrated with 0.05 M Tris acetate/0.01 M EDTA (pH 8.1). The liposomes emerging in the exclusion peak still contained 0.17 mol potassium ferricyanide per mol phospholipid. The trapped solute was reducible by a crude NADH-cytochrome b_5-reductase (which also reduces potassium ferricyanide) only in the presence of deoxycholate. In an identical experiment without alkali treatment we found 0.78 mol trapped solute per mol phospholipid. These data show that short alkali treatment rendered protein-carrying liposomes more leaky than the control samples, but did not completely destroy the vesicular structure. Therefore ionization experiments with lipo-cytochrome b_5 seemed feasible.

The ionized tyrosine residues were determined by the absorbance difference between 295 nm and 315 nm, 295 nm being the absorbance maximum of ionized tyrosine in the difference spectrum [23] and 315 nm being the nearest minimum at higher wavelengths. In several experiments with cytochrome d-b_5 and lipo-cytochrome b_5 the maximal value of $A_{295\text{nm}} - A_{315\text{nm}}$ agreed well with the theoretical value for the ionization of 5 tyrosines, whereas in other experiments it was too high. The titrations reported here were reversible; upon neutralization of the alkaline solutions $A_{295\text{nm}} - A_{315\text{nm}}$ returned practically to zero. Huntley and Strittmatter [21] described a similar discrepancy with lipase-extracted cytochrome b_5 which they attributed to changes in the heme spectrum at high pH. But when we carried out ionization experiments with another heme-containing protein, cytochrome c, this assumption could not be verified. We currently are not able to explain the occasional differences between the theoretical and experimental values of $A_{295\text{nm}} - A_{315\text{nm}}$. But since the ratio of the initial values of $A_{295\text{nm}} - A_{315\text{nm}}$ to the final values was reproducible in the different forms of cytochrome b_5, we calculated the degree of ionization at a given pH from the ratio of $A_{295\text{nm}} - A_{315\text{nm}}$ at t = 1 min to the final value of $A_{295\text{nm}} - A_{315\text{nm}}$ at pH 13. The latter should correspond to the ionization of all tyrosines. Fig. 3 shows the ionization curves of cytochrome d-b_5 and lipo-cytochrome b_5 at t = 1 min and t = 180 min.

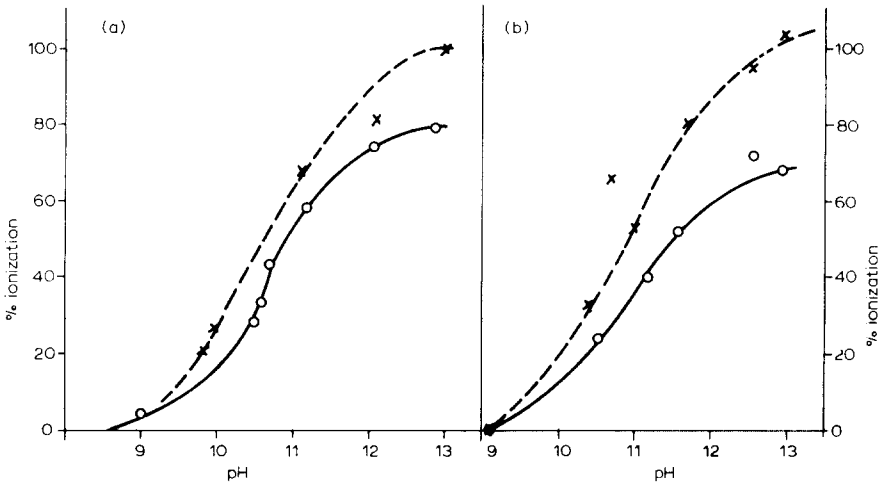

Fig. 3. Ionization curves of cytochrome *d*-b_5 and lipo-cytochrome b_5. Difference spectra between 270 nm and 340 nm were recorded of solutions of increasing pH versus neutral solutions. Percentage ionization was calculated from the ratio of $A_{295nm} - A_{315nm}$ at a given pH to $A_{295nm} - A_{315nm}$ at t = 180 min and pH 13 multiplied by 100. (a) ionization curves of 10.6 μM cytochrome *d*-b_5. (b) ionization curves of 13.2 μM lipo-cytochrome b_5. —o——o—, ionization curves obtained at t = 1 min; —X——X—, ionization curves obtained at t = 180 min.

It is evident that 180 min after the addition of alkali the titration curves of cytochrome b_5 and its liposomal form become identical. By that time, presumably, the lipid vesicles are destroyed. After 1 min, however, 20% of the tyrosines in cytochrome *d*-b_5 have not yet reacted, while in lipo-cytochrome b_5 35% are not yet ionized. Table I contains the average values obtained for all three forms of the cytochrome.

Since all 3 tyrosines in cytochrome *t*-b_5 ionize immediately and since the tertiary structure of that part of the molecule appears to have the same conformation in cytochrome *t*-b_5, cytochrome *d*-b_5 and lipo-cytochrome b_5 [24], 81% ionization for cytochrome *d*-b_5 means that half of the two tyrosine residues in the hydrophobic peptide are protected against OH^-. In lipo-cytochrome b_5, these two tyrosines are protected almost completely.

TABLE I

THE IONIZATION OF THE TYROSINES IN CYTOCHROME *t*-b_5, CYTOCHROME *d*-b_5, AND LIPO-CYTOCHROME b_5 AFTER 1 MIN AT PH 13

4—15 μM protein solutions were brought to pH 13 with 1 M KOH and difference spectra of the alkaline versus neutral solutions were determined at t = 1 min and at 45-min intervals up to 180 min. Percentage ionization is calculated as the ratio of $A_{295nm} - A_{315nm}$ at t = 1 min to the final value of $A_{295nm} - A_{315nm}$, multiplied by 100.

Sample	Ionization (%)	Number of experiments
Cytochrome *t*-b_5	100 *	3
Cytochrome *d*-b_5	81 ± 4 **	3
Lipo-cytochrome b_5	63 ± 6 **	3

* Final ionization determined at t = 120 min.
** Final ionization determined at t = 180 min.

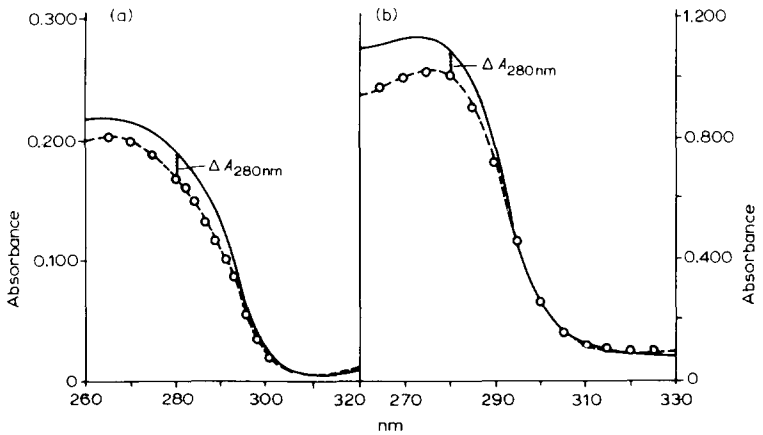

Fig. 4. The deacetylation of acetylated lipo-cytochrome b_5 and cytochrome d-b_5 by hydroxylamine. (a) 3.7 μM cytochrome d-b_5 and (b) 13 μM lipo-cytochrome b_5 in 0.01 M Tris · HCl (pH 7.5) were acetylated with acetylimidazole and freed from excess reagent by passage through Sephadex G 25. The ultraviolet spectra of the acetylated proteins were recorded and 1/4 vol 2.5 M hydroxylamine (pH 7.5) was added to the solutions. After 1 h at room temperature, the ultraviolet spectra were recorded once more. The ultraviolet spectra of the acetylated proteins were corrected for the change of volume caused by the addition of hydroxylamine. —o——o—, spectrum of acetylated protein; ———, spectrum of deacetylated protein.

Acetylation of the tyrosines. The ionization experiments seemed to point to one tyrosine of the hydrophobic peptide which in lipo-cytochrome b_5 ionizes less readily than in cytochrome d-b_5. Because of the drastic conditions of these experiments, we also used acetylation with *N*-acetylimidazole as another modification method. The acetylation proceeds under very mild conditions, that is at pH 7.5 in 0.01 M Tris · HCl at room temperature, and deacetylation is effected in 0.5 M hydroxylamine, again at neutral pH. Under these conditions, all 5 tyrosines in cytochrome d-b_5 as well as in lipo-cytochrome b_5 were accessible to acetylimidazole and hydroxylamine, as could be seen by the increase of the absorbance at 280 nm upon addition of hydroxylamine to the acetylated protein (Fig. 4). Acetylation did not affect the Soret peak at 413 nm indicating that the immediate environment of the heme group was not changed by this procedure.

(c) The oxidation of tryptophan by N-bromosuccinimide

Of the four tryptophan residues in cytochrome b_5, three are located in the hydrophobic "root". Owing to this distribution, the tryptophans are especially well suited as probes of the reactivity of the hydrophobic region in the lipid-free and lipid-bound state. However, treatment of cytochrome d-b_5 with *N*-bromosuccinimide in acid or neutral solutions did not result in any changes at 280 nm. Only after the unfolding of the protein in 8 M urea all four tryptophans were accessible to the oxidant. The tryptophan present in cytochrome t-b_5 was oxidized under the same conditions. In the case of lipo-cytochrome b_5, preliminary experiments were carried out to ascertain that the lipoprotein was not disrupted in acidic urea solutions. Lipo-cytochrome b_5 was dissolved in 8 M urea at pH 4 or 5. Filtration through Sephadex G 200 or Controlled Pore Glass CPG-10-1000 Å resulted in a first peak containing protein and phos-

pholipid in the same ratio as the original lipoprotein whereas the pure protein was trapped and could not be eluted. We also examined the influence of the reagent on protein-free liposomes and found that treatment of liposomes in acidic urea solutions with *N*-bromosuccinimide did not change the absorbance at 280 nm.

The stability of lipid vesicles in concentrated urea was tested with liposomes containing a trapped solute. Cytochrome *d*-b_5 was bound to liposomes containing potassium ferricyanide. These were incubated for 80 min with 8 M urea (pH 6.6) at room temperature. After passage through Sephadex G 25 they still contained 0.75 mol potassium ferricyanide per mol phospholipid which were enzymatically reducible only in the presence of deoxycholate. Control liposomes retained 0.78 mol potassium ferricyanide per mol phospholipid. This experiment shows that even in the presence of protein high concentrations of urea do not noticeably damage the stability of the lipid vesicles.

Exposure of lipid-bound cytochrome b_5 to *N*-bromosuccinimide in 8 M urea resulted in the modification of up to 60% of the tryptophan residues. The pH of the reaction mixture did not significantly influence this value: at pH 4 the average was 53%, at pH 6.6 it was 60%. At pH 4, the deviation from the average was higher than at pH 6.6. Fig. 5 shows the ultraviolet spectra of the original cytochrome *d*-b_5 and lipo-cytochrome b_5 solutions in 8 M urea, pH 4, and of the modified preparations.

The spectroscopic data indicate that 40% of the tryptophans in lipo-cytochrome b_5 are inaccessible to the reagent. This could be confirmed by amino acid analysis of an alkaline hydrolysate of modified lipo-cytochrome b_5 which showed about 40% of the tryptophans to be still intact. We also prepared the liposome-bound hydrophobic peptide by digesting lipo-cytochrome b_5 with trypsin and isolating the lipid-bound part by gel filtration through Sephadex G 200. Treatment of this liposome-bound peptide with *N*-bromosuccinimide at pH 4 resulted in the oxidation of 53% of the tryptophan residues. The results of these experiments are presented in Table II.

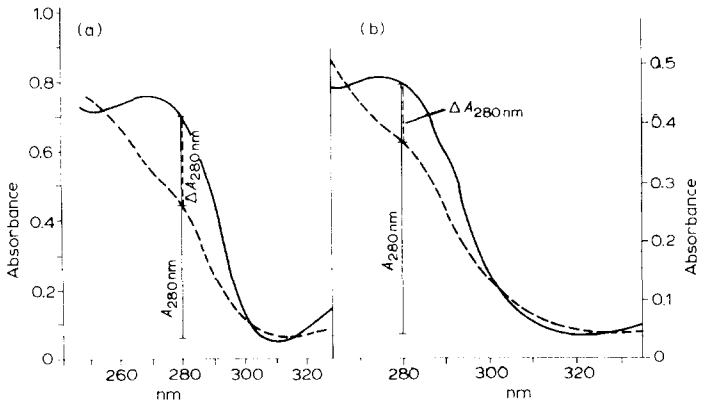

Fig. 5. The oxidation of tryptophan residues in lipo-cytochrome b_5 and cytochrome *d*-b_5 by *N*-bromosuccinimide. (a) 8.2 μM lipo-cytochrome b_5 and (b) 14 μM cytochrome *d*-b_5 in 8 M urea (pH 4) were titrated with 9 mM *N*-bromosuccinimide, until the absorption at 280 nm remained constant. Ultraviolet spectra were recorded of the untreated (———) and the oxidized (- - - - -) solutions.

TABLE II

THE OXIDATION OF THE TRYPTOPHAN RESIDUES IN DIFFERENT FORMS OF CYTOCHROME b_5 BY *N*-BROMOSUCCINIMIDE

Samples in 8 M urea were treated with increasing amounts of *N*-bromosuccinimide until the absorption at 280 nm remained constant. The percentage of tryptophan residues modified was calculated from the decrease in the absorption at 280 nm. The figures in parentheses give the number of experiments.

Sample	Tryptophan	Tryptophan oxidized (%)	
	cytochrome b_5	at pH 4	at pH 6.6
Cytochrome *t*-b_5	1	70 ± 15 (2)	—
Cytochrome *d*-b_5	4	93 ± 8 (4)	93 (1)
Lipo-cytochrome b_5	4	53 ± 13 (7)	60 ± 5 (4)
Hydrophobic peptide bound to liposomes	3	53 ± 13 (4)	—

Consumption of *N*-bromosuccinimide depended on pH. At pH 4, 4—5 mol of oxidant were used for the oxidation of 1 mol tryptophan in cytochrome *d*-b_5 as well as in lipo-cytochrome b_5. At pH 6.6, about 7 mol of reagent were consumed per mol of tryptophan oxidized, presumably because of side reactions with the heme.

Discussion

In contrast to cytochrome *t*-b_5, cytochrome *d*-b_5 is oligomeric in aqueous buffers [1]. But according to the model proposed by Strittmatter [2], and later confirmed by others [4,25], cytochrome b_5 exists as a monomer in the microsomal membrane. The question arises whether oligomeric cytochrome *d*-b_5 can be considered as "native" and whether its reactivity can be compared to that of the monomeric lipo-cytochrome b_5. Since the object of these experiments was to study the influence of the lipid bilayer on the reactivities of tyrosine and tryptophan residues, it was not possible to use cytochrome *d*-b_5 depolymerized by attachment to another kind of vesicle such as deoxycholate micelles as control. The study on tryptophan reactivity involved treatment with high concentrations of urea which partly depolymerize cytochrome *d*-b_5 [1]. But in the case of tyrosine, the reactivities of the lipid-free protein were determined on the oligomer. It was conceivable that the tyrosines, while exposed to the solvent in lipo-cytochrome b_5, might be buried in the free protein as a consequence of protein-protein interactions, resulting in a lower reactivity in the free state compared to the lipid-bound state.

This was found not to be the case. The ionization experiments seemed to show immediate ionization of all three tyrosines in cytochrome *t*-b_5 at pH 13. Of the two tyrosines in the root segment, 50% do not immediately ionize at pH 13 even in lipid-free cytochrome *d*-b_5. In lipo-cytochrome b_5, these tyrosines are shielded to at least 80%. The data cannot reveal whether both tyrosines are equally inaccessible to OH^- or whether one tyrosine is completely buried and the other partly exposed. It could be shown that the bilayer structure persists in alkali for short periods of time, therefore the results of the ionization experiments would be consistent with the assumption that part of the hydrophobic domain containing a tyrosine becomes inaccessible upon binding to a lipid

membrane. However, the results of the acetylation experiments seem to rule out this possibility. All tyrosines are easily acetylated and the acetylated tyrosines are accessible to hydroxylamine. Therefore, the different ionization behaviour of the tyrosines in the root peptide presumably reflects differences in the tertiary structure of the hydrophobic segment in the aggregated and the lipid-bound form.

Differences in tertiary structure cannot account for the results of the tryptophan modification experiments. Oxidation with *N*-bromosuccinimide occurs only after denaturation of the molecule with 8 M urea. The native conformation has to be destroyed in order to render the tryptophans accessible to the reagent which in concentrated urea solutions is not *N*-bromosuccinimide, but *N*-bromourea [26].

The unresponsive tryptophan residues therefore belong to regions which are protected by the lipid bilayer against unfolding. That the lipid bilayer remains stable in concentrated urea solutions was demonstrated by our experiments with liposomes containing trapped potassium ferricyanide. Dufourcq et al. in their study on tryptophan fluorescence of cytochrome b_5 in the presence of lipids [27] arrived at the same conclusion, namely that the fluorescent tryptophans are in contact with the fatty acid chains of the phospholipids. These studies also confirm that lipid-protein interactions persist even in 8 M urea. Fluorescence measurements, however, do not tell whether all three tryptophans of the hydrophobic segment are in the interior of the lipid phase.

Our experiments with the chemical modification of the tryptophan side chains reveal different kinds of exposure. About 40% of the tryptophans do not react with *N*-bromourea. This means that about half of the tryptophan side chains in the hydrophobic part are shielded by the lipid bilayer. Considering the results from the fluorescence studies, at least one tryptophan must be completely embedded in the hydrophobic interior of the lipid bilayer. The remaining two tryptophans are either both partly shielded from the attack by *N*-bromourea, they might be located in the vicinity of the polar headgroups of the phospholipids, or one of them is partly shielded and the other fully exposed to the solvent.

The data presented here demonstrate that the hydrophobic segment is indeed embedded in the lipid matrix of the membrane. They also show that by no means all of it is immersed in the lipid bilayer. The exposed parts may be adjacent to the hydrophilic domain or they may belong to the end of the polypeptide chain. They may also belong to single loops protruding from the bilayer. At the moment, it is not possible to distinguish between these possibilities.

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